



Simian Immunodeficiency Virus DNA Vaccine Trial in Macaques

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An experimental vaccine consisting of five DNA plasmids expressing different combinations and forms of simian immunodeficiency virus-macaque (SIVmac) proteins has been evaluated for the ability to protect against a highly pathogenic uncloned SIVmac251 challenge. One vaccine plasmid encoded nonreplicating SIVmac239 virus particles. The other four plasmids encoded secreted forms of the envelope glycoproteins of two T-cell-tropic relatives (SIVmac239 and SIVmac251) and one monocyte/macrophage-tropic relative (SIVmac316) of the uncloned challenge virus. Rhesus macaques were inoculated with DNA at 1 and 3, 11 and 13, and 21 and 23 weeks. Four macaques were inoculated intravenously, intramuscularly, and by gene gun inoculations. Three received only gene gun inoculations. Two control monkeys were inoculated with control plasmids by all three routes of inoculation. Neutralizing antibody titers of 1:216 to 1:768 were present in all of the vaccinated monkeys after the second cluster of inoculations. These titers were transient, were not boosted by the third cluster of inoculations, and had fallen to 1:24 to 1:72 by the time of challenge. Cytotoxic T-cell activity for Env was also raised in all of the vaccinated animals. The temporal appearance of cytotoxic T cells was similar to that of antibody. However, while antibody responses fell with time, cytotoxic T-cell responses persisted. The SIVmac251 challenge was administered intravenously at 2 weeks following the last immunization. The DNA immunizations did not prevent infection or protect against CD4⁺ cell loss. Long-term chronic levels of infection were similar in the vaccinated and control animals, with 1 in 10,000 to 1 in 100,000 peripheral blood cells carrying infectious virus. However, viral loads were reduced to the chronic level over a shorter period of time in the vaccinated groups (6 weeks) than in the control group (12 weeks). Thus, the DNA vaccine raised both neutralizing antibody and cytotoxic T-lymphocyte responses and provided some attenuation of the acute phase of infection, but it did not prevent the loss of CD4⁺ cells.

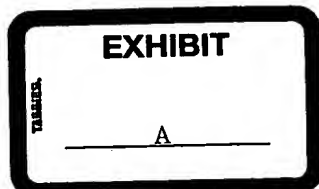
The human immunodeficiency virus (HIV) has proved to be difficult to vaccinate against (5). One central problem in HIV vaccine development is the antigenic diversity of patient isolates (32). A second central problem is the poor ability of postinfection immune responses to protect against the slow but inexorable development of AIDS. A successful vaccine will need to prevent a broad spectrum of serologically distinct viruses from establishing a foothold infection. To achieve this, the vaccine may be required to maintain high levels of continuously active immune responses that are capable of controlling a diversity of HIV type 1 (HIV-1) isolates.

Primate models for immunodeficiency virus vaccine trials use HIVs (HIV-1 and HIV-2), simian immunodeficiency viruses (SIVs) (such as SIVmac, SIVmne, and SIVsm) (25), and hybrids of SIVs and HIVs (SHIVs) (35, 58, 61). Challenge infections with these viruses range from avirulent to highly virulent. In avirulent challenges, such as HIV-1 in chimpanzees, HIV-2 in macaques, or SHIVs in macaques, most infected primates naturally control the infection and do not develop disease. In these benign models protective immunization has been achieved with recombinant vectors, envelope subunits, and even envelope peptides (for examples, see refer-

ences 21, 51, 52, and 60). In models of intermediate virulence, such as SIVmne infections in pig-tailed macaques, animals develop disease but only after a latency period of at least 1 year. In these intermediate models the combination of recombinant vaccinia virus vectors plus subunit boosts has been required for protection (26, 27). In yet more pathogenic models, such as molecularly cloned SIVmac239 or uncloned SIVmac251 infections in rhesus macaques, substantial numbers of test animals develop AIDS during the first year of infection. In these highly pathogenic models only live attenuated infections have generated protective immunity (2, 11). Thus, the success of different vaccine approaches has depended on the virulence of the challenge infection.

No common correlate for protection has been identified in the various vaccine trials. Neutralizing antibody has been a correlate for protection for neutralization-susceptible challenges, such as those with HIV-1-IIIb Env proteins (4, 21, 30), but not for "difficult-to-neutralize" viruses, such as uncloned SIVmac251 or molecularly cloned SIVmac239 (12, 19, 30, 42). Cytotoxic T lymphocytes (CTL) appear to contribute to protection. This is suggested by the observation that the combination of internal viral proteins and envelope glycoproteins is more effective than envelope glycoproteins alone at raising protection (27, 73). The nature of the protective responses raised by live attenuated infections is not clear. For a neutral-

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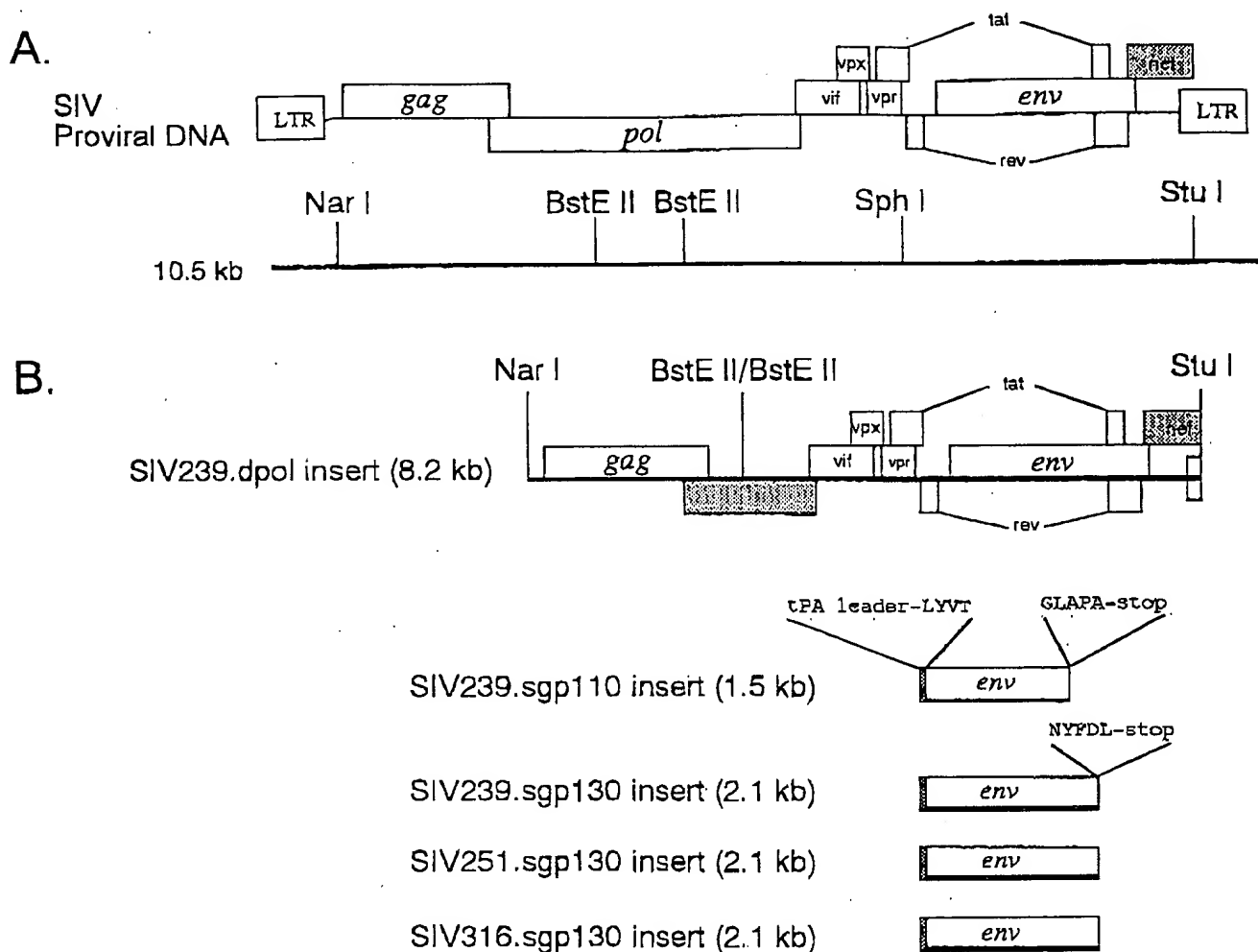


FIG. 1. Vaccine inserts. (A) SIV239 proviral DNA; (B) vaccine inserts. SIV239 open reading frames are indicated as open rectangles, closed or defective reading frames are indicated as dotted rectangles, and the tissue plasminogen activator (tPA)-like leader is indicated as a filled rectangle. Restriction sites used for the construction of the SIV239.dpol insert are indicated. For the constructs expressing secreted forms of Env, the amino acid sequences at the junctions of the tPA-like leader and introduced stop codons are indicated. The SIV239.dpol insert was expressed in pCMV/BC12. The Env inserts were expressed in pJW4303. See Materials and Methods for details. LTR, long terminal repeat.

ization-susceptible challenge (SIVsmB670), protection has correlated with the raising of neutralizing antibody (8). For difficult-to-neutralize viruses, protection has required active replication of the vaccine virus, with the extent of protection correlating with the level of vaccine replication (36, 39). Thus, protection by live attenuated vaccines may involve suppressive effects raised by chronic immunodeficiency virus infections in addition to antibody and CTL responses.

Recently, a new approach to vaccination has opened up with the demonstration that antigen-expressing plasmid DNAs can raise protective immunity (9, 14, 18, 38, 56, 63, 69, 71). This novel method of vaccination elicits both cellular and humoral immunity. Furthermore, DNA-mediated immune responses have shown the long-lived effector activity (41, 55, 62, 72) which may be essential to an AIDS vaccine.

Relatively limited work has been done using DNA to raise immune responses to immunodeficiency viruses. In murine models, plasmids expressing the HIV-1 envelope glycoprotein (Env) have raised both neutralizing antibody and CTL responses (17, 37, 68). DNA inoculations in macaques have also raised antibody to HIV-1 Env (66) and have achieved some reduction in postchallenge load with a SHIV challenge (67).

In this study we have undertaken an initial evaluation of the ability of DNA-expressed antigens to protect against an immunodeficiency virus challenge. The highly virulent uncloned SIVmac251-rhesus macaque model was chosen for the trial to test whether DNA could achieve the protection provided by a live attenuated vaccine (11). The uncloned SIVmac251 challenge virus is a difficult-to-neutralize stock that causes $\geq 50\%$ incidence of AIDS during the first year of infection.

MATERIALS AND METHODS

Vaccine DNAs. The pSIV239.dpol vaccine DNA was created by cloning the SIV239.dpol insert into the pBC12/CMV expression vector. pBC12/CMV uses approximately 750 bp from the cytomegalovirus immediate-early promoter to drive transcription and sequences from the rat preproinsulin II gene to provide a 3' intron and polyadenylation signal (10). The SIV239.dpol insert (Fig. 1) was generated from two separate plasmids representing the 5' (p239SpSp5') and 3' (p239SpE3') halves of a 239 provirus with a premature stop codon in *nef* (11, 21). The 5' half was rendered *pol* defective by deletion of a 754-bp *BstEII* fragment (bp 3571 to 4325). This deletion removed 251 codons in *pol* and introduced a stop codon at the religated *BstEII* site. *NarI* was used to truncate long terminal repeat sequences in the 5' proviral piece, and *StuI* was used to truncate long terminal repeat sequences in the 3' piece. A *NarI*-to-*SphI* fragment of the *pol*-defective 5' proviral fragment, an *SphI*-to-*StuI* fragment of the 3' half,

and a blunt *Bam*HI-to-*Hind*III fragment of pBC12/CMV were ligated together to produce pSIV239.dpol.

The other four vaccine plasmids were constructed by cloning PCR-amplified fragments of SIV *env* sequences (Fig. 1) into the pJW4303 expression vector. pJW4303 is modeled on vectors described by Chapman et al. (7). It uses approximately 1,600 nucleotides from the cytomegalovirus immediate-early promoter (nucleotides 458 to 2063; GenBank accession number M60231) to drive transcription and sequences from the bovine growth hormone (nucleotides 2148 to 2325; GenBank accession number M57764) to provide a polyadenylation signal. The vector includes a synthetic mimic of the tissue plasminogen activator leader sequence (ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGCTCTTCGTTTCGCTAGC) that can be placed in frame with expressed proteins by using an *Nhe*I site (underlined in the sequence). Templates used for PCR amplifications included p239SpE3' (SIV239), pAbT4593 (SIV251), and pL239E/V-3 (SIV316) (31, 48). The 5' PCR primer was designed to clone *env* fragments in frame with the tissue plasminogen activator leader by using the *Nhe*I site. The 3' primers were designed to introduce stop codons for the production of secreted gp110 (sgp110) or gp130 (sgp130) forms of Env and to facilitate cloning into the *Bam*HI site of pJW4303. The 5' primer used for constructions was japer19 (GTCGCTCCAAGCTTGTCTAGC CAATATGTACAGCTCTTTTATGG) (the *Nhe*I site is underlined). The 3' primer for sgp110 was hpcr2 (CCCGGGATCGATCGCGGCGCCAGGC CAATCGGAGTGATCTCTACTAATTT), and that for sgp130 was jw8 (CGG GATCGATCGCGGCGCCAGGTCACCAATTCGCTAGC) (the *Bam*HI site is underlined and the stop codon is in lowercase in the primer sequences). Amplification was carried out in a 100- μ l reaction mixture with 2 μ g of purified plasmid, 30 pmol of each primer, and 200 μ M deoxynucleoside triphosphates. Five cycles of amplification were used with 10 U of cloned *Pfu* polymerase in the buffer provided by the manufacturer (Stratagene, La Jolla, Calif.). Each cycle consisted of 94°C for 45 s, 56°C for 60 s, and 72°C for 120 s. Samples were ethanol precipitated, gel purified, digested with *Nhe*I and *Bam*HI, and ligated into *Nhe*I- and *Bam*HI-digested pJW4303. Control plasmid DNAs consisted of pBC12/CMV (pCMV/control) and pJW4303 without inserts. Vaccine and control plasmids were grown in the HB101 strain of *Escherichia coli* and purified twice on cesium chloride density gradients by standard protocols.

Expression of vaccine DNAs. In vitro expression of vaccine DNAs was tested by protein blot analyses of transiently transfected COS cells. Purified plasmid DNAs were transfected into COS cells with a Gene Pulser (Bio-Rad, Hercules, Calif.) at 960 μ F and 250 V. Two days later, cells were lysed with 10 mM Tris buffer containing 1% Triton X-100, and cell debris were removed by microcentrifugation at 12,000 rpm for 1 h at 4°C. Aliquots of cell lysates were diluted 1:1 in 2 \times sample buffer (0.125 M Tris-HCl, 4.6% sodium dodecyl sulfate [SDS], 10% 2-mercaptoethanol, 20% glycerol, pH 6.8), boiled for 2 min, cooled, and loaded onto a 10% polyacrylamide gel for SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were electrophoretically transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, Mass.). The membrane was blocked with 5% nonfat dried milk in 20 mM Tris buffer (pH 7.5) and then incubated with SIV-infected monkey sera (1:300 dilution in Tris buffer). Finally, the membrane was incubated with 5 μ Cl of ¹²⁵I-protein G in 0.5% milk-Tris buffer for 1 h, washed with Tris buffer, air dried, and subjected to autoradiography.

COS cells transfected with pSIV239.dpol or pCMV/control were examined for the production of virus-like particles by electron microscopy. At 48 h posttransfection, cells were removed from plates with TEN buffer (40 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 7.5), pelleted in a microcentrifuge tube, and fixed in 1% glutaraldehyde. Cells were prepared for thin-section electron microscopy by using osmium tetroxide (70).

Monkeys. Six young adult female and three young adult male rhesus monkeys (*Macaca mulatta*), which were negative for antibodies to SIV, simian retrovirus D, simian T-cell leukemia virus type 1, and herpes simplex virus type 1, were randomly assigned to two vaccine groups and one control group. The animals were individually housed and cared for at TSI Mason Inc. (Worcester, Mass.) according to approved standard operating procedures. Animals were tranquilized for inoculations and blood collections by using ketamine-HCl (10 mg/kg) as needed. Cageside observations were performed twice daily throughout the trial. Physical examinations, including a record of general condition, body weight, rectal body temperature, and heart rate, were performed at the initiation of the experiment, at weeks 11, 21, and 25 prior to virus challenge, and monthly after challenge.

Saline and gene gun inoculations of DNA. Saline injections consisted of 500 μ g of each DNA dissolved in 0.5 ml of saline. Intravenous inoculations were administered by mixing all of the DNAs to be delivered (0.5 ml per DNA) and performing a single injection into the brachial vein. Intramuscular injections delivered 0.25 ml of each DNA to a separate site in each quadriceps. Each gene gun inoculation consisted of 1.44 mg of 1- to 3- μ m-diameter gold beads that had been coated with 7.2 μ g of DNA by precipitation of the DNA onto beads in the presence of spermidine and Ca²⁺ (16). Gene gun inoculations were administered to areas of the abdominal skin (ca. 10 by 15 cm) and areas of skin on the inner thigh (ca. 5 by 10 cm). Target areas were prepared by shaving and then removing stubble with the depilatory agent *Noir* (Carter Products, New York, N.Y.). A hand-held electric-discharge Accell particle bombardment device (Agracetus Inc., Middleton, Wis.) was used for gene gun inoculations. Histologic analyses of

tissue from target areas revealed the highest density of beads in the epidermal layer of the skin.

Virus challenge. Challenge was by intravenous inoculation into the brachial vein of 10 monkey infectious doses, in 1 ml of tissue culture medium, of uncloned SIVmac251 grown in monkey peripheral blood mononuclear cells (PBMC) (34).

Assays for neutralizing antibody with the neutralization-susceptible stock. Neutralizing antibodies to SIVmac251 and SIVsmB670 were measured in a virus-induced cell-killing assay performed in 96-well microdilution plates as described previously (45), except that CEMx174 cells were used as targets for infection. Briefly, 100 50% tissue culture infective doses of virus was incubated with various dilutions of sera for 30 min at 37°C before the addition of cells. Neutralization was quantified by staining viable cells with neutral red. Neutral red uptake measured by A_{540} is linear from 0.25 to 1.6, corresponding to 3.1×10^4 to 2.5×10^5 viable cells per well (45). Percent protection was calculated by the difference in A_{540} between test wells (cells plus serum sample plus virus) and virus control wells (cells plus virus) divided by the difference in A_{540} between cell control wells (cells only) and virus control wells. Assay mixtures were harvested when the cytopathic effect in virus control wells was greater than 70% but less than 100%. Neutralizing titers were defined as the reciprocal of the serum dilution that protected 50% of cells from virus-induced killing. The 50% tissue culture infective dose was calculated as described previously (45).

Neutralization-susceptible stock. SIVmac251 and SIVsmB670 stocks for neutralization assays were harvested from cultures of acutely infected H9 cells. Stocks were clarified by low-speed centrifugation and filtration through 0.45- μ m-pore-size cellulose-acetate filters (Millipore) and stored at -70°C until used. The stocks of SIVmac251 and SIVsmB670 used to determine titers of neutralizing antibodies are sensitive to neutralization in several assays (29, 54).

Assays for neutralizing antibody with the challenge stock. Neutralization of the challenge stock was tested on human PBMC and CEMx174 cells. Neutralization of SIV in human PBMC was measured essentially as described previously for HIV-1 (43). Briefly, cell-free virus (10 to 20 ng of p27 per ml) was incubated with diluted test plasmas at 37°C for 1 h in triplicate wells of 96-well U-bottom plates before addition of CEMx174 cells or phytohemagglutinin-stimulated PBMC (10^5 cells in 50 μ l added per well). Heat-inactivated plasmas were evaluated at a 1:30 dilution by incubating 50 μ l of virus with 100 μ l of plasma that had been diluted 1:20 in growth medium. An additional six wells containing no test sample (control wells) were included to determine the kinetics of virus replication in the absence of neutralizing antibodies. The plates were incubated for 3 h at 37°C, after which the cells were washed extensively with growth medium to remove the virus inoculum and test serum. Cell suspensions (25 μ l) were collected every day beginning on day 2 and mixed with 225 μ l of 0.5% Triton X-100, and virus production was quantified by p27 immunoassay as described by the supplier (Coulter Immunology, Hialeah, Fla.). Virus replication in control wells was linear from day 3 through day 8, during which time p27 production increased from approximately 1 to 25 ng/ml. Virus production in test wells was measured at a time when production in control wells was in the range of 8 to 10 ng of p27 per ml. We have found that measurements of neutralization made during this stage of virus replication in control wells provide a high level of sensitivity and reproducibility to the assay. A positive score in this assay is $\geq 90\%$ reduction of p27 CA production. These assays are more sensitive for the scoring of neutralization than the assay used for neutralization-susceptible stocks.

Growth of challenge stock for neutralization assays. Uncloned SIVmac251 was obtained as a frozen vial of rhesus PBMC-grown animal challenge stock that had been titrated in rhesus macaques (34). Virus taken from this vial was used directly to inoculate rhesus PBMC to generate an expanded single-passaged stock that was used for in vitro assays. Expansion was performed by incubating cells with cell-free virus for 1 day, removing the virus inoculum by a series of washes, and then incubating the cells in fresh growth medium. Culture fluids were collected every 2 days, passed through 0.45- μ m-pore-size filters, and stored at -70°C in 1-ml aliquots. A frozen aliquot from each time point was thawed, and SIV core antigen was quantified by p27 CA immunoassay (Coulter Immunology). Culture fluids that contained the highest virus p27 concentrations were used for neutralization assays.

SIVmac251 gp110 ELISA. SIVmac251 gp110-specific immunoglobulin G was measured by enzyme-linked immunosorbent assay (ELISA) with alkaline phosphatase-conjugated goat anti-monkey immunoglobulin G (whole molecule; Sigma Chemical Company, St. Louis, Mo.) as described previously (54). SIVmac251 gp110 was a generous gift from Kashi Javaherian (Repligen Corporation, Cambridge, Mass.). Titers are reported as the reciprocal of the highest serum dilution giving an average A_{405} of greater than 0.1 and at least twice that of a negative control serum from a normal, healthy, SIV-negative rhesus macaque.

Assay for anti-CD4 antibodies. To test for anti-CD4 antibodies in monkey sera, uninfected CEM cells (2×10^5 /ml) were stained with a 1:10 dilution of the test serum for 30 min at room temperature in 100 μ l of phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% sodium azide (PBS-BSA-NaAz). The cells were washed with PBS-BSA-NaAz and stained with 10 μ l of fluorescein isothiocyanate-conjugated anti-monkey immunoglobulin G (whole molecule; Organon-Teknica-Cappel, Durham, N.C.). The cells were washed a final time and then suspended in 2 ml of PBS-BSA-NaAz, after which fluorescence was measured with a Coulter XL MCL flow cytometer. Fluorescence intensities were determined relative to those of cells stained with only the second

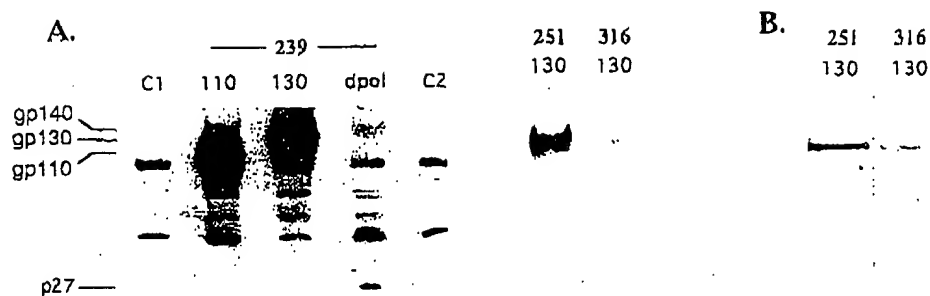


FIG. 2. Expression of vaccine DNAs. Protein blot analyses of lysates (A) and culture medium (B) from transiently transfected COS cells are shown. Approximately 1/5 of the cell lysate and 1/100 of the culture supernatant were loaded. Numbers above the lanes indicate the SIVmac isolate from which inserts were prepared (239, SIVmac239; 251, SIVmac251; 316, SIVmac316) and the expressed insert (110, *sgp100*; 130, *sgp130*; *dpol*, noninfectious particles). C1 and C2, lysates of COS cells transfected with a control vector without an insert. The positions of SIV Env (*gp140*, *gp130*, and *gp110*) and CA (*p27*) proteins are indicated on the left. SIV proteins were detected by using serum from an SIV-infected macaque as the first antibody. For details, see Materials and Methods.

antibody. Serum from a macaque immunized with human cell-grown SIV and containing high-titer anticell antibodies was used as a positive control.

C-ADE assay. Complement-mediated, antibody-dependent enhancement (C-ADE) of SIVmac251 infection was measured by testing for virus production in MT-2 cells by a cross-reactive HIV-1 p24 antigen capture ELISA (44). C-ADE is given as the titer (last serum dilution to show enhancement), peak (serum dilution producing the greatest increase in p24 production), and power (p24 production at the peak divided by p24 production in the absence of test serum or complement). Measurements of the titer and peak utilize a minimum twofold increase in p24 production as a cutoff value. The neutralization stock of SIVmac251 (see above) was used for C-ADE assays.

CTL assays. Effector cells for CTL assays were antigen-restimulated peripheral blood lymphocytes (65). To prepare effector cells, aliquots of 10^7 peripheral blood lymphocytes were placed in 12-well plates with 10^7 stimulator cells (see below). This cell mixture was cultured in 2 ml of RPMI 1640 medium containing 20% fetal calf serum (Flow Laboratories, McLean, Va.). On day 3 of culture, 2 ml of medium containing recombinant interleukin-2 (40 U/ml) (provided by Hoffman-La Roche, Nutley, N.J.) was added to each culture. At day 6 of culture, dead cells were removed by Ficoll-Paque density gradient centrifugation, and the viable cells were assayed for cytotoxic function.

Stimulator cells were prepared by infecting B-lymphoblastoid cell lines with recombinant vaccinia virus expressing SIVmac *gag* or *env* or the irrelevant equine herpesvirus 1 *gH* gene at a multiplicity of infection of 10 for 12 h (65). Infected cultures were washed, and 10^7 viable cells were resuspended in 5 ml of 1.5% paraformaldehyde in PBS for 30 min at room temperature, pelleted, resuspended in 5 ml of 0.2 M glycine-PBS for 15 min at room temperature, and then maintained in fetal calf serum at 4°C until used in culture.

Effector cells were assayed for CTL activity on autologous B-lymphoblastoid target cells (65). Target cells were prepared by incubation for 16 h at 37°C in a 5% CO₂ atmosphere with both recombinant vaccinia virus expressing SIVmac *gag* or *env* or the irrelevant equine herpesvirus 1 *gH* gene and 0.5 mCi of Na₂⁵¹CrO₄ (ICN, Irvine, Calif.) per ml. Effector cells were incubated with ⁵¹Cr-labeled target cells for 5 h with effector-to-target cell ratios of 20:1, 10:1, 5:1, and 3:1. Specific release was calculated as [(experimental release - spontaneous release)/(100% release - spontaneous release)] × 100. Spontaneous release varied from 10 to 20%.

Postchallenge tests for infection. Postchallenge levels of antigenemia were determined by analysis of plasma by the Coulter p27 CA antigen capture ELISA. Postchallenge levels of infected PBMC were determined by limiting-dilution cocultivations. PBMC from 10 ml of whole blood were separated with Ficoll-Paque gradients. Twelve serial 1:3 dilutions of PBMC, beginning with 10^6 cells, were cocultured in duplicate with 10^5 CEMx174 cells per well in a 24-well plate in 1 ml of RPMI 1640 supplemented with 10% fetal bovine serum. After 3 to 4 days of culture, 1 ml of medium was added to each well. The cultures were then split at 3- to 4-day intervals with an equal volume of fresh medium. Supernatants were collected at 21 days and stored at -70°C. Supernatant samples were assayed for p27 antigen by the Coulter antigen capture ELISA. The virus load was calculated as the minimum number of monkey PBMC required to infect 50% of the coculture wells.

Test for CD4⁺ cells. CD4⁺ cells were quantitated by fluorescence-activated cell sorting of OKT4a (Ortho Diagnostics, Raritan, N.J.)-stained PBMC. These are scored as the percentage of the prechallenge level. The prechallenge levels of CD4⁺ cells were established by averaging the results of analyses conducted with three independent harvests of cells from prechallenge animals.

RESULTS

Vaccine DNAs and verification of expression. A mixture of plasmid DNAs was constructed for use in vaccination (Fig. 1). The first of these, pSIV239.dpol, was designed to express noninfectious SIVmac239 particles. The purpose of the SIV239.dpol DNA was to present a broad spectrum of SIVmac proteins. pSIV239.dpol was constructed from the same sequences as used for the construction of the *nef*-defective SIV239 virus, which had successfully protected against an uncloned SIV251 challenge (11). Sequences from both LTRs as well as sequences from an internal region of *pol* were deleted to reduce the potential for the generation of a replication-competent retrovirus.

Four vaccine plasmids were constructed to express envelope glycoproteins of two T-cell-tropic derivatives (SIVmac239 and SIVmac251) and one monocyte/macrophage-tropic derivative (SIVmac316) of the uncloned SIV251 challenge virus (6, 59). Three of these Env-expressing vaccine DNAs, designated SIV239.sgp130, SIV251.sgp130, and SIV316.sgp130, expressed the entire extracellular domain of Env (Fig. 1B). The fourth, SIV239.sgp110, expressed the receptor binding subunit of Env (Fig. 1B). The secreted envelope glycoproteins were expressed as fusion proteins with a synthetic tPA leader sequence. This allows Env expression in the absence of Rev (7).

Verification of the expression of the vaccine DNAs was accomplished by analyses of transiently transfected COS cells (Fig. 2 and 3). Protein blots of pSIV239.dpol-transfected cells revealed the expression of several SIV proteins (Fig. 2A). Electron micrographs demonstrated the production of particles by the SIV239.dpol insert (Fig. 3). Protein blots of cells transfected with the Env-expressing DNAs revealed the expected forms of Env in the culture medium as well as in the cell lysates (Fig. 2). Most of the glycoprotein expressed by the *sgp130*-expressing plasmids appeared to be present as an ~130 kDa band, suggesting that proteolytic cleavage of the *sgp130* form of Env at the SU/TM boundary is inefficient in COS cells. SIV-specific proteins and virus-like particles were not observed in cells transiently transfected with control DNA.

Trial design. The trial consisted of two vaccine groups and one control group (Table 1). The first vaccine group was vaccinated by gene gun, intravenous, and intramuscular inoculations. The second vaccine group was inoculated by gene gun only. The control group received control plasmid DNAs without inserts by all three routes. Gene gun inoculations were used for all of the monkeys because gene gun delivery of DNA



FIG. 3. Electron micrograph demonstrating particle production by the SIV239.dpol vaccine DNA in transiently transfected COS cells. Bar, 100 μ m.

to the epidermis has proved to require relatively low doses of DNA to raise responses (18, 49, 55, 69). Intramuscular and intravenous saline injections of DNA were used in one vaccine group to test the abilities of these routes of inoculation to

augment responses raised by gun inoculations of skin. Because of the lower efficiency of saline injections, larger amounts of DNA were used for intramuscular and intravenous inoculations than for the gun inoculations (18, 49, 55, 69).

The DNA inoculation schedule consisted of three clusters of two inoculations each (spaced at 2 weeks) with intervening 8-week rest periods (Fig. 4). Clustered inoculations were used because such inoculations can enhance antibody responses to low levels of administered proteins (15). In the first cluster of inoculations, the vaccine groups received SIV239.dpol DNA, SIV239.sgp110 DNA, and SIV239.sgp130 DNA. For the second and third clusters of inoculations, both vaccine groups received the three 239 DNAs plus gene gun-delivered SIV251.sgp130 and SIV316.sgp130 DNAs (Table 1).

An intravenous challenge with 10 animal infectious doses of uncloned SIV251 was administered 2 weeks following the final DNA boost. This challenge time was chosen because optimal antibody responses are present at 2 weeks following immunization with protein subunits (for examples, see references 3 and 22).

Antibody responses in DNA-vaccinated macaques. Both ELISA responses and neutralizing antibodies were raised in all of the vaccinated monkeys (Fig. 5). The first cluster of DNA inoculations raised ELISA responses in two of the seven vaccinated monkeys (one in each group). The second cluster of inoculations was followed by ELISA responses and neutralizing activity in all of the monkeys. Similar titers of neutralizing activity were raised in the gene gun and multiple-route groups.

TABLE 1. Summary of DNAs administered in different groups

Group	No. of monkeys	DNA(s)	Dose	Route	Dosing frequency
1	4	239.dpol 239.sgp120 239.sgp140	250 μ g each	Intramuscular injections at two sites for each plasmid	Once at wk 1, 3, 11, 13, 21, and 23
			500 μ g each	Intravenous injection	
		239.dpol ^a 239.sgp120 239.sgp140	7.2 μ g each	Gene gun in two abdominal sites and one site in each thigh for each plasmid	
			7.2 μ g each	Gene gun in two abdominal sites for each plasmid	
2	3	239.dpol ^a 239.sgp120 239.sgp140	7.2 μ g each	Gene gun in two abdominal sites and one site in each thigh for each plasmid	Once at wk 1, 3, 11, 13, 21, and 23
			7.2 μ g each	Gene gun in two abdominal sites for each plasmid	
		251.sgp140 316.sgp140	7.2 μ g each	Gene gun in two abdominal sites for each plasmid	Once at wk 11, 13, 21, and 23
3	2	pCMV/ control pJW4303	250 μ g (pCMV/control) and 500 μ g (pJW4303)	Intramuscular injections at two sites (pCMV/control) or four sites (pJW4303)	Once at wk 1, 3, 11, 13, 21, and 23
			500 μ g (pCMV/control) and 1.0 mg (pJW4303)	Intravenous injection of indicated plasmids	
		pJW4303	7.2 μ g each	Gene gun in two (pCMV/control) and four (pJW4303) abdominal sites and in one (pCMV/control) and two (pJW4303) sites in each thigh	
			7.2 μ g each	Gene gun in four abdominal sites	Once at wk 11, 13, 21, and 23

^a Gene gun administration of 239.dpol consisted of 4.8 μ g of dpol DNA and 2.4 μ g of pCrev DNA.

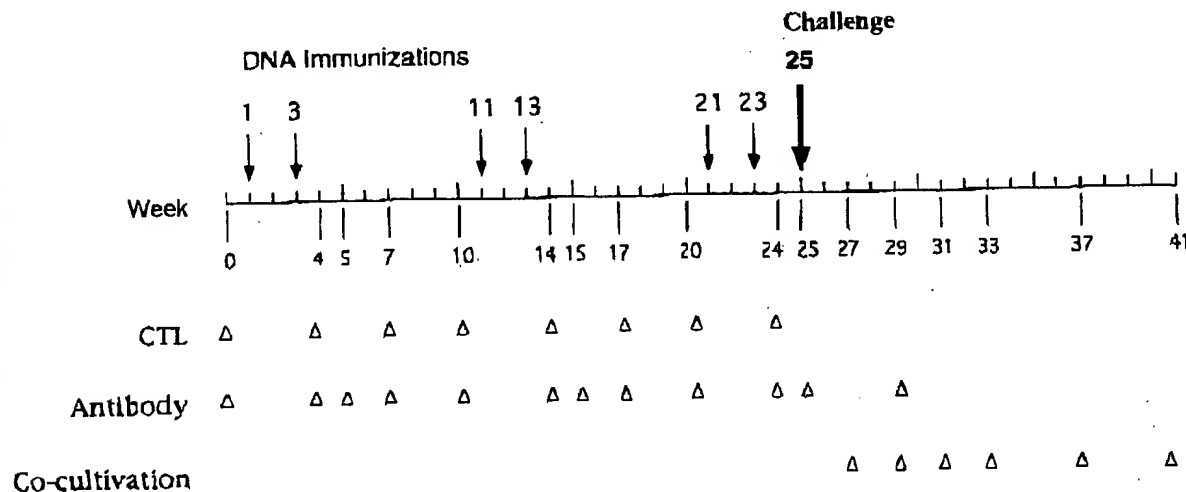


FIG. 4. Design of the DNA vaccine trial.

Anti-Env antibody responses were transient, with the titers of both ELISA and neutralizing activities falling between the second and third clusters of DNA inoculations. The third cluster of inoculations boosted the ELISA titers to levels similar to those achieved after the second cluster of inoculations but failed to boost the neutralizing antibody titers, which continued to fall. To test whether the falling titers of neutralizing antibody reflected the appearance of anti-idiotypic antibody resembling the receptor binding site on Env, sera taken at weeks 14, 25, and 29 were tested for the ability to react with uninfected CD4⁺ cells. These tests were negative (data not shown), suggesting that the loss of neutralizing antibody was not caused by the appearance of anti-idiotypic antibody. The falling titers of neutralizing antibody also could not be attributed to the raising of enhancing antibodies (which could obscure neutralizing activity), because enhancing antibodies do not score in the neutralization assay.

The two control monkeys had low levels of ELISA activity for SIVmac251 gp110, which increased over time (Fig. 5). Peak ELISA titers for one of the controls, 8R7, correlated with low levels of neutralization activity (Fig. 5). This may reflect a generalized and nonspecific activation of humoral responses by large amounts of plasmid DNA (approximately 18 mg per monkey in groups receiving DNA by three routes).

Susceptibility of the challenge stock and an unrelated SIV (SIVsmB670) to raised antibody. Peak titers of neutralizing antibody were tested for their ability to neutralize the uncloned SIVmac251 challenge (Table 2). These assays were conducted with 1:30 dilutions of sera on CEMx174 cells (a cell line that is favorable for the scoring of neutralizing activity) as well as on human PBMC. Sera were compared with sera obtained from macaques chronically infected with uncloned SIVmac251. None of the sera had significant neutralizing activity in the assay on PBMC. The more sensitive assay on CEMx174 cells scored low levels of activity in most of the DNA-raised sera. A serum pool from chronically infected macaques also showed neutralizing activity in the assay on CEMx174 cells.

The prechallenge sera were also assayed for the ability to neutralize SIVsmB670. Each of the tested sera showed good activity against this unrelated immunodeficiency virus (Table 2). In 7 of 8 vaccinated animals, the ratio of neutralizing activity for SIVsmB670 to that for SIVmac251 was higher than

that in sera pooled from 12 chronically infected macaques. This suggests that the use of multiple different envelope genes in the vaccine had raised neutralizing activity with good cross-reactivity for neutralization-susceptible stocks.

CTL responses in DNA-vaccinated macaques. CTL responses to Env were raised in all of the monkeys receiving vaccine DNAs (Fig. 6). These responses were detected following antigen restimulation of peripheral blood lymphocytes. Following the first cluster of inoculations, anti-Env CTL were present in three of the seven vaccinated monkeys. One of these was in the group receiving DNA by three routes of inoculation, and two were in the group receiving only gene gun inoculations. After the second cluster of inoculations, all of the vaccinated monkeys exhibited anti-Env CTL activity. The levels of CTL activity were not further increased by the third cluster. In contrast to the anti-Env antibody responses, which fell with time, the anti-Env CTL responses were persistent (Fig. 5 and 6).

Definitive CTL responses for Gag were seen in only two of the vaccinated animals (Fig. 6). Both of these monkeys were in the group receiving multiple routes of DNA inoculation. One monkey (L44) had high CTL responses to Gag following the first cluster of inoculations. This was the same monkey that had high antibody responses following the first cluster of inoculations (Fig. 5).

The CTL activity for Env and Gag was both CD8 lymphocyte mediated and major histocompatibility complex restricted (74). Monkeys receiving control DNA did not have anti-Env or anti-Gag cytolytic activity (Fig. 6).

Postchallenge levels of infection. Challenged animals were tested for protection against infection by examining antigenemia at 2 weeks postchallenge. The vaccinated animals had 4- to 100-times-lower levels of CA in their plasma than the control animals, with three of the vaccinated animals scoring below the lowest point on the standard curve (Fig. 7). The detection of CA in the vaccinated animals indicated that the vaccine had not prevented infection. The low levels of CA in the vaccinated animals could have reflected early control of the infection by the vaccine and/or interference of vaccine-induced anti-CA antibody with the antigen capture ELISA.

To more accurately evaluate levels of infection, PBMC from the challenged animals were tested for the frequency of infected cells in a limiting-dilution cocultivation assay (Table 3).

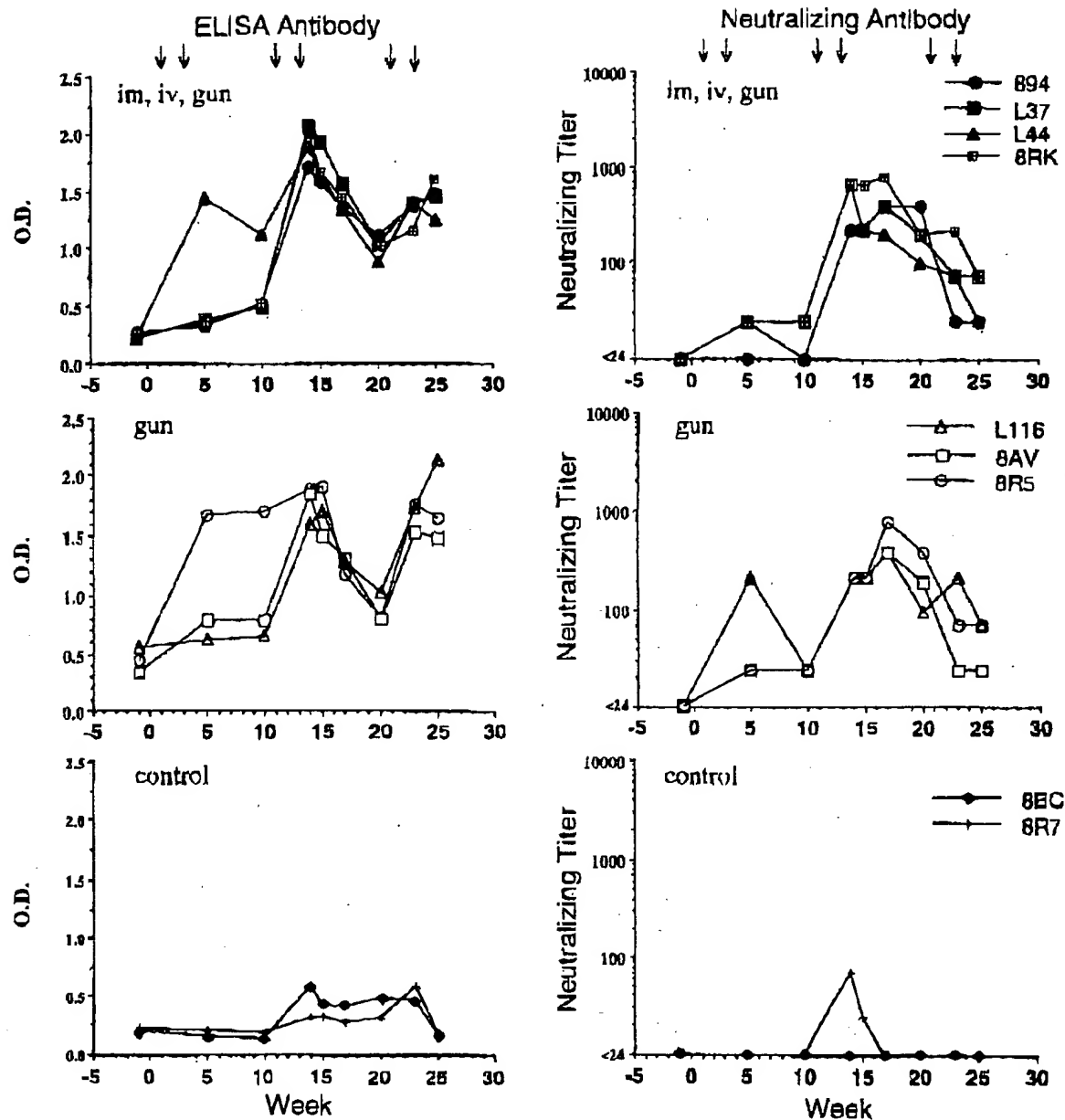


FIG. 3. Temporal antibody responses in vaccine and control groups. Left panels, ELISA antibody for SIVmac251 gp116; right panels, neutralizing antibody for the neutralization stock of SIVmac251. The times of DNA administration are indicated by vertical arrows at the top of the schematic. Test groups: im, iv, gun, group receiving intramuscular, intravenous, and gene gun inoculations of vaccine DNA; gun, group receiving only gene gun inoculations of DNA; control, group receiving control plasmid DNAs by intramuscular, intravenous, and gene gun inoculations. Symbols for the individual monkeys are given at the right. Assays for ELISA antibody were run on 1:50 dilutions of test sera (O.D., optical density). Neutralizing titers are the reciprocals of the highest dilutions of sera giving neutralization of 100 50% tissue culture infective doses of the neutralizable stock of SIV251 on CEMx174 cells. For more details, see Materials and Methods.

At 2 weeks postchallenge, these assays revealed similar frequencies of infected PBMC in the vaccinated and control animals. However, at 4 weeks postchallenge, all of the vaccinated animals had lower viral loads than at 2 weeks postchallenge, whereas only one of the two control animals had reduced its viral load. Again, at 6 weeks postchallenge, all of the vaccinated animals had still lower levels of infected lymphocytes, while only one of the two control animals had decreased its

level of infected PBMC. At this time, the overall difference in the levels of infected PBMC in the vaccine and control groups was about fivefold. Thus, the vaccination appeared to achieve some acceleration in the reduction of viral loads. The kinetics of this reduction were similar in both of the vaccinated groups.

Despite the early reductions in viral load, the vaccinated animals did not clear their infections. Furthermore, with time, the control group achieved reductions in viral load similar to

TABLE 2. Neutralizing activities of peak titers of DNA-raised sera for SIVmac251, SIVsmB670, and the uncloned SIVmac251 challenge stock^a

Group	Monkey	Titer ^b		Neutralization for challenge stock ^c on:	
		SIVmac251	SIVsmB670	CHMx174 cells	Hutum PDMC
Gene gun, intramuscular, and intravenous administration	L44	216	216	0.5	0.9
	K94	216	216	0.1	0.9
	L37	216	216	0.1	0.7
	RRK	648	216	0.08	0.6
Gene gun administration	L116	216	216	0.2	1.0
	RR5	216	216	0.3	0.8
	RAV	216	216	0.1	0.8
Control	BDC	<24	<24	0.8	0.8
	RR7	24	<24	1.0	0.7
SIVmac251-infected macaques	CMP-1 ^d	5,832	1,944	<0.03	0.8

^a Sera were from week 15 of the trial.^b The titer is the reciprocal of the highest serum dilution giving 50% neutralization.^c Assays were conducted at 1:30 dilutions of sera. Values are p27 production for assays with a test serum divided by p27 production for assays with a normal macaque serum. Sera are considered positive for neutralization if the production of p27 C/A was reduced by more than 90%. See text for details.^d The CMP-1 serum was pooled from 12 chronically infected macaques.

those seen in the vaccinated macaques (Table 3, week 12). Thus, the DNA vaccination did not allow the vaccinated animals to achieve viral loads lower than those achieved by postinfection responses in the control animals.

Antibody responses at 1 month postchallenge. Despite the failure of the last cluster of DNA inoculations to boost neutralizing activity (Fig. 5), the challenge infection raised high titers of neutralizing antibody. At 1 month postchallenge, these titers in the vaccinated groups were similar to or slightly higher than those in the control group (Table 4).

Interestingly, each of the vaccinated animals also had high titers of neutralizing activity for SIVsmB670 (Table 4). These titers were 3 to 27 times higher than the titers for SIVmac251. In contrast, the control animals had similar or lower levels of neutralizing activity for SIVsmB670 (Table 4; see also Table 2). This is in agreement with the vaccination regimen having primed a neutralizing response broader than that present in most naturally infected animals.

Postchallenge titers of ELISA antibody for Env were much higher (~100 times) in the vaccinated groups than in the control group (Table 4). This difference presumably reflects the vaccine having primed nonneutralizing as well as neutralizing antibodies for Env.

Complement-dependent antibody enhancement. Complement-dependent enhancing antibodies could be detected in the sera of the vaccinated animals prechallenge and in the sera of the vaccinated and control animals postchallenge (Table 5). On the day of challenge, both of the vaccinated groups had similar titers of enhancing antibody. These titers increased postchallenge. At 1 month postchallenge, the vaccinated and control animals had similar titers of complement-dependent enhancing antibodies. This suggests that the higher ELISA response and broader neutralizing response of the DNA-vaccinated animals was not accompanied by higher titers of enhancing responses (Tables 4 and 5).

Postchallenge CD4 cell levels and mortality. Consistent with the failure to achieve long-term reductions in viral loads, all of the vaccinated animals exhibited steadily declining levels of CD4⁺ cells (Fig. 8). One of the control animals maintained steady CD4⁺ levels despite an active SIV infection (Fig. 7 and 8; Tables 3 and 4).

The trial was terminated at 1 year postchallenge. At this time, the three macaques in the gene gun-only group and one of the two control macaques (the one with the steady CD4⁺ level) had succumbed to AIDS (Fig. 8). The second control monkey and the four monkeys in the multiple-route group did not have clinical signs of AIDS at the time of euthanasia.

DISCUSSION

The DNA vaccine raised both CTL and antibody responses but failed to protect against the highly virulent uncloned SIVmac251 challenge. Persistent CTL responses were raised, with easily detected levels of activity being present at the time of challenge (Fig. 6). Only transient titers of neutralizing antibody were raised (Fig. 5). These failed to be boosted by the third cluster of inoculations and had fallen 10-fold by the time of challenge. The DNA-raised CTL and antibody responses did not prevent infection (Table 3). DNA-raised anamnestic responses were evident in distinct patterns of postchallenge antibody responses in the vaccinated and control groups (Table 4). Anamnestic responses may have provided an early period of more effective virus clearance (Fig. 7 and Table 3) but failed to prevent the loss of CD4⁺ cells (Fig. 8). The goal of future trials will be to improve this window of protection.

CTL and antibody responses: similar temporal appearances but better persistence of CTL response. Interestingly, the kinetics of CTL and antibody responses were similar, whereas the persistences of these responses were different (Fig. 5 and 6). Both CTL and antibody responses appeared in some monkeys following the first cluster of inoculations and in all animals following the second cluster of inoculations. The early antibody responders were also the early CTL responders. Thus, the raising of both antibody and CTL was concordant. However, the anti-Env antibody responses were transient, while anti-Env CTL responses persisted. This is consistent with different immunological mechanisms supporting the long-term persistence of effector cells for antibody and CTL.

The transience of anti-Env antibody responses in macaques is in keeping with an earlier HIV-1 DNA vaccine trial in mice (37). Transient antibody responses have also been seen in mice

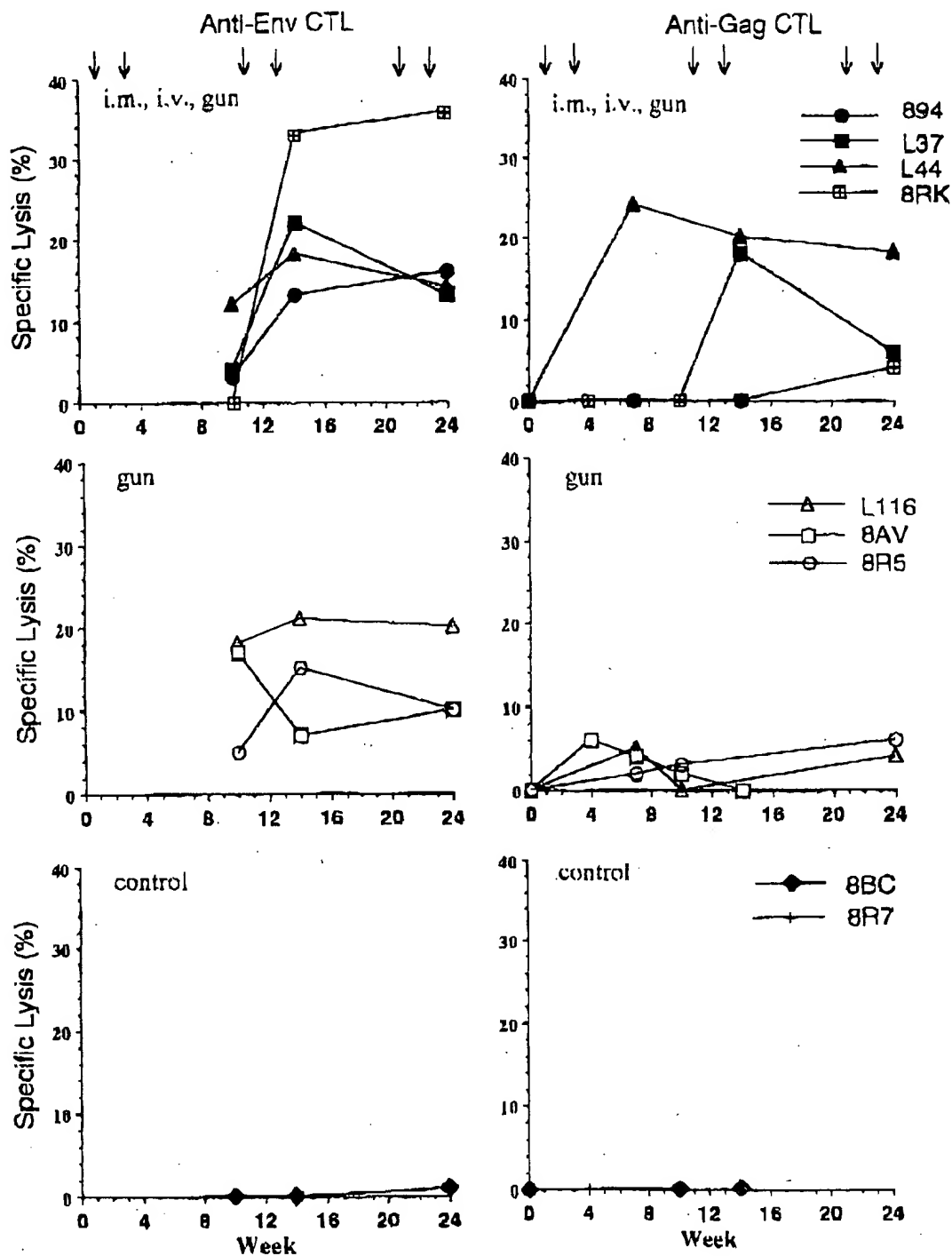


FIG. 6. Temporal CTL responses in vaccine and control groups. Anti-Env and anti-Gag CTL responses are for antigen-restimulated lymphocytes from peripheral blood. Arrows, designations of test groups, and symbols for individual monkeys are as in Fig. 5. Specific lysis is given for an effector-to-target cell ratio of 20:1. Levels of specific lysis below 5% were not considered significant. (For more details, see Materials and Methods.)

immunized with DNA expressing the circumsporozoite protein of malaria (47). These transient humoral responses are in contrast to the persistent antibody responses seen in mice immunized with DNAs expressing the influenza virus hemagglutinin and nucleoprotein (53, 55), the hepatitis B virus sur-

face antigen (41), the rabies virus glycoprotein G (71), and HIV-1 CA (37). DNA-raised humoral responses to influenza virus HA and NP proteins also persist in African green monkeys (14). Differences in the persistence of DNA-raised responses have been independent of the method of DNA inoc-

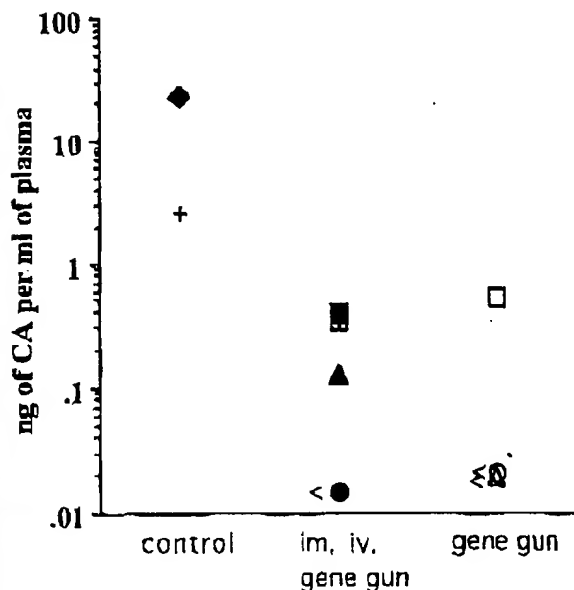


FIG. 7. Antigenemia at 2 weeks postchallenge. Symbols for monkeys and designations of test groups are the same as in Fig. 5 and 6. Values preceded by \leq were between the lowest point of the standard curve and the background level.

ulation (Fig. 5) (37, 55). Thus, the persistence of antibody appears to be a property of the expressed antigen.

The temporal persistence of CTL responses in this study does not agree with the results of a prior study in BALB/c mice, in which antibody responses appeared to down-regulate CTL responses (17). This difference may reflect differences in the regulation of T-helper responses in macaques (an outbred population) and BALB/c mice (an inbred line). The differences could also be contributed to by the more frequent use of boosts in the BALB/c mouse study.

Ability of DNA to prime broad anti-Env responses. The DNA vaccine primed for a broader neutralizing response than observed in most naturally infected animals (Tables 2 and 4). This may reflect the use of a mixture of DNA-encoded Env proteins. Alternatively, this could be contributed to by re-

peated immunizations with small amounts of antigen (as expressed by DNA), serving to broaden responses (23).

The ability to use DNA to raise broad neutralizing responses is important to the development of an AIDS vaccine. Envelope glycoproteins of laboratory strains of HIV-1 have been ineffective at raising neutralizing antibody for patient isolates (for a review, see reference 46). The ability to use DNA to raise neutralizing antibody will facilitate the evaluation of whether envelope sequences, or mixtures of envelope sequences from patients, can be used to raise cross-reactive neutralizing activity for primary isolates.

Enhancing antibody. In addition to raising neutralizing and ELISA activities, the DNA inoculations elicited complement-dependent enhancing antibody (Table 5). The enhancing activity (which was present at the time of challenge) could have contributed to the failure of the vaccine to prevent infection. By 1 month postchallenge, similar titers of enhancing antibody were present in the vaccinated and control macaques (Table 5). Therefore, the vaccine regimen did not prime for higher titers of enhancing antibody than raised by the natural infection. One goal of future studies is to use DNA to evaluate whether the deletion of enhancing sequences in Env can support the raising of neutralizing, but not enhancing, antibody (57).

Enhancing antibodies were scored on the neutralization-susceptible stock but not the challenge stock. Enhancing assays on the neutralization stock of SIV251 and molecularly cloned SIVmac239 (a difficult-to-neutralize virus) suggest that different variants of SIVmac will not show the differences in susceptibility to enhancing antibody that are seen for susceptibility to neutralization activity (42). This may reflect a higher level of conservation of epitopes that are targets for enhancement than of epitopes that are targets for neutralization (57).

Better CTL activity for Env than for Gag. All of the DNA-vaccinated macaques developed CTL for Env, whereas only two developed CTL for Gag (Fig. 6). This could reflect a difference in the levels of Gag and Env expression in the vaccinated macaques. All five of the vaccine DNAs expressed Env, whereas only one (SIV239.dpol) expressed Gag (Fig. 1). The difference in the incidence of Env and Gag CTL could also reflect a higher proportion of SIV-infected macaques mounting CTL responses to Env than to Gag (64).

DNA immunizations: comparison with live attenuated vaccinations. Our aim in undertaking this trial was to use DNA

TABLE 3. Postchallenge levels of infected PBMC

Group	Macaque	Infected PBMC (% of total) at postchallenge wk ^a :			
		2	4	6	12
Gene gun, intramuscular, and intravenous administration	L44	0.04	0.02	0.001	0.008
	R94	0.1	0.04	0.0005	0.01
	L37	0.3	0.04	0.008	0.01
	NRK	0.7	0.1	0.01	0.01
	Avg	0.3	0.05	0.006	0.01
Gene gun administration	L116	0.01	0.01	0.004	0.008
	RR5	0.3	0.01	0.003	0.0009
	HAV	0.7	0.1	0.01	0.02
	Avg	0.4	0.04	0.006	0.01
Control	ABC	0.1	0.1	0.02	0.02
	RR7	0.2	0.04	0.04	0.003
	Avg	0.2	0.07	0.03	0.01

^a Limiting-dilution co-cultivations of PBMC with CEMa174 indicator cells were used to determine the percentages of infected PBMC at various times postchallenge. For details, see Materials and Methods.

TABLE 4. Neutralizing and ELISA antibodies at 1 month postchallenge

Group	Macaque	Neutralizing antibody			ELISA antibody	
		Titer ^a		Ratio (8670/251) ^b	251 titer ^c	251 neu/ELISA ^d
		SIVmac251	SIVsmB670			
Gene gun, intramuscular, and intravenous administration	L44	1,944	5,832	3	8,100	0.2
	894	5,832	17,496	3	8,100	0.7
	L37	5,832	17,496	3	24,300	0.2
	HRK	5,832	17,496	3	24,300	0.2
Gene gun administration	L116	5,832	17,496	3	24,300	0.2
	RR5	5,832	52,488	9	8,100	0.7
	8AV	648	17,496	27	8,100	0.8
Control	8BC	1,944	216	0.1	100	19
	8R7	1,944	1,944	1	100	19

^a Values are the reciprocal of the highest dilution giving 50% neutralization of 100 50% tissue cultures infective doses for neutralization stocks of SIVmac251 and SIVsmB670.

^b Titer of neutralizing antibody for SIVmac251 divided by titer of neutralizing antibody for SIVsmB670.

^c Titer of ELISA antibody for recombinant SIV251 gp110. Titers are the highest reciprocal serum dilution having an average absorbance reading greater than 0.1 and at least twice that of the negative control.

^d Titer of neutralizing antibody for SIVmac251 divided by titer of ELISA antibody for SIV251 gp110.

inoculations to determine if they could provide the protection achieved by a live attenuated vaccine. Both transfected and infected cells express antigens in cells, allowing access to class I major histocompatibility antigens. Proteins expressed by transfected cells are like proteins expressed by infected cells in undergoing normal patterns of glycosylation. The ability to use a mixture of DNAs can be used to mimic the mixture of viruses present in an uncloned virus challenge.

Our results demonstrate that the DNA inoculations and protocol used in this trial fell short of raising the responses achieved by live attenuated infections. First and foremost, the

DNA inoculations failed to provide protection (Fig. 8). However, vaccinations in this trial were conducted over a 6-month period (Fig. 4), whereas it is now known that live attenuated infections may require as long as 1 year to develop protective responses (13). Perhaps a longer period of DNA immunization would have supported the "maturation" of a protective response. The DNA vaccine was also unlike live attenuated infections in failing to raise persisting titers of neutralizing antibody (Fig. 5) (11). This failure may not have affected the outcome of the trial because prechallenge titers of neutralizing antibody have not correlated with protection against the uncloned SIVmac251 challenge (12) (see the introduction). Insufficient information is available to compare the CTL responses raised by live attenuated infections with those raised in this study (Fig. 6). In future trials we hope to be able to directly compare the cell-mediated responses raised by DNA and live attenuated vaccines to learn how these differ. It is also possible that the persistent replication of a live attenuated virus raises suppressive responses that are not provided by DNA-based antigenic stimulation.

Better survival in monkeys receiving vaccine DNA by three routes of inoculation. The three monkeys in the gene gun-only group developed opportunistic infections (AIDS) prior to the termination of the trial, whereas the four monkeys receiving DNA by three routes did not (Fig. 8). This difference in survival did not correlate with differences in antibody and CTL responses (Fig. 5 and 6), differences in levels of postchallenge infection (Table 3), or differences in the rates of CD4⁺ cell decline (Fig. 8). Thus, the difference in survival could have been due to chance. Alternatively, it might reflect gene gun and saline vaccinations having different consequences for the ability of infected animals to resist opportunistic infections.

Dosing schedule. The current trial used a series of three clusters of DNA inoculations with intervening 8-week rest periods (Fig. 4). In mice and rabbits, longer rest periods (4 to 5 months) have resulted in better boosting of anti-Env antibody (unpublished data). Four- to 5-month rest periods have also been found to enhance the effects of protein boosts in primates (3, 22). Also, we no longer use clustered inoculations, because they do not appear to have the marked effect on efficacy observed for clustered inoculations of low levels of protein (15).

TABLE 5. Complement-dependent enhancing antibody

Group	Macaque	Wk	C-ADL ^a		
			Titer	Peak	Power
Gene gun, intramuscular, and intravenous administration	L44	-1	<30	<30	<2.0
		14	30	30	4.9
		25	30	30	4.9
	L37	29	>3,840	30/60	5.4
		-1	<30	<30	<2.0
		14	30	30	3.3
	HRK	25	30	30	3.5
		29	>3,840	240	4.0
		-1	<30	<30	<2.0
		14	240	30	5.2
		25	960	30	6.0
		29	>3,840	30	4.8
Gene gun administration	L116	-1	<30	<30	<2.0
		14	30	30	4.3
		25	120	30	5.1
		29	>3,840	30	4.5
Control	8BC	25	<30	<30	<2.0
		29	>3,840	120	5.1
	8R7	25	<30	<30	<2.0
		29	>3,840	30	5.2

^a Complement-dependent enhancing activity was determined on MT-2 cells. See Materials and Methods for experimental details.

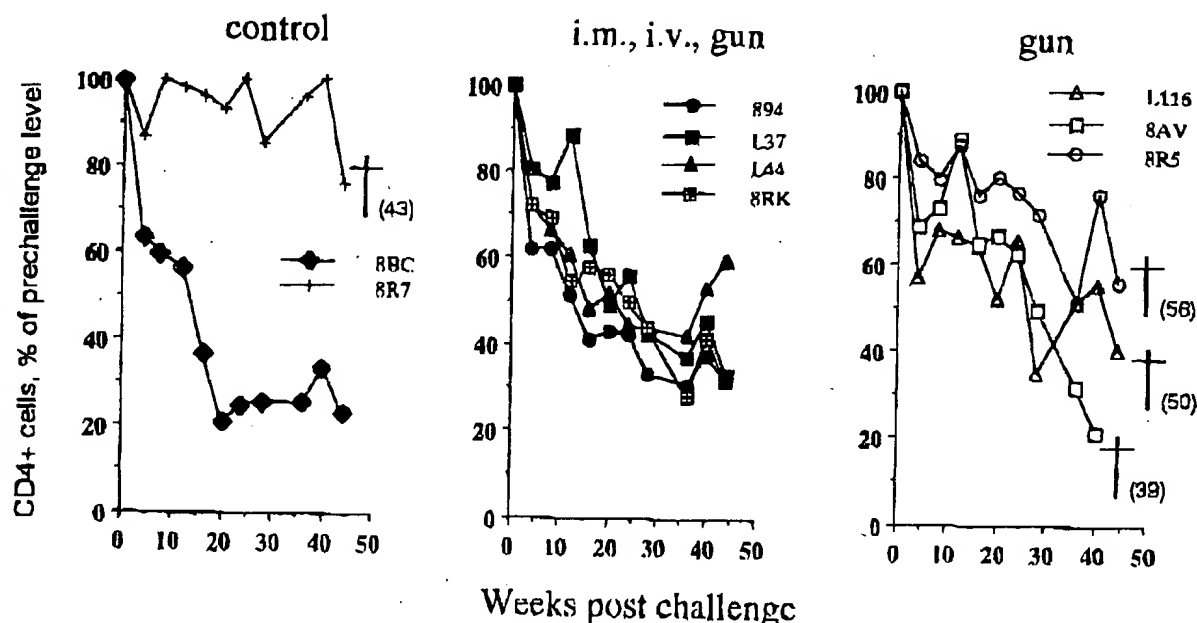


FIG. 8. Temporal levels of CD4⁺ cells in challenged monkeys. The designations of groups and symbols for monkeys are the same as in Fig. 5 to 7. The daggers followed by numbers in parentheses designate the week of euthanasia for macaques that developed AIDS. Absolute numbers of CD4⁺ cells per microliter of blood used as the baseline values for the animals were 836 for 8AV, 989 for L37, 1,117 for L44, 1,153 for 8BC, 1,222 for 8RK, 1,435 for 1.116, 1,441 for 8R5, 1,717 for 894, and 2,159 for 8R7. For more details, see Materials and Methods.

Thus, DNA-based immunization appears to obey some, but not all, of the principles which govern immunization with proteins.

Atypical CD4⁺ cell profile in one macaque. One of the two control macaques maintained high levels of CD4⁺ cells despite an active SIV infection (Fig. 8; Tables 3 and 4). Serological tests of this monkey were negative for simian T-cell leukemia virus. We do not understand this atypical preservation of CD4⁺ cells or have a sufficient database to know how frequently this phenomenon occurs in SIVmac251-infected macaques.

Protection of a population as opposed to protection of the vaccinated individual. Historically, vaccination has protected the vaccinated individual against the development of disease. However, vaccines have also protected populations by reducing viral shedding. In this as well as prior trials, vaccines that have failed to prevent the development of disease have attenuated the acute phase of infection (Table 3) (1, 20, 24, 28, 36, 40). Vaccines that curtail the acute phase of infection reduce transmission by reducing the window of time in which an individual has a high virus load. AIDS vaccines that attenuate the acute phase of infection are much more achievable than vaccines that prevent AIDS. These should be given serious consideration for populations experiencing the rapid spread of AIDS.

Role of DNA in the future development of immunodeficiency virus vaccines. The success with which DNA can raise antibody and CTL responses in monkeys shows that DNA is a viable addition to current approaches to AIDS vaccines (66, 67; this study). Improvement in DNA-raised immune responses should be easily achieved by improvements in vectors and vaccination protocols. Improved responses can also be achieved by combining DNA priming with protein or recombinant vaccinia virus boosts (reference 33 and data not shown). On the basis of our current experience, our belief is that DNA-based immuni-

zations will support the development of a successful AIDS vaccine.

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